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UTILITY PATENT APPLICATION TRANSMITTAL (Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.
ISPH-0518

Total Pages in this Submission

TO THE ASSISTANT COMMISSIONER FOR PATENTS

Box Patent Application
Washington, D.C. 20231

Transmitted herewith for filing under 35 U.S.C. 111(a) and 37 C.F.R. 1.53(b) is a new utility patent application for an invention entitled:

ANTISENSE MODULATION OF TNFR1 EXPRESSION

and invented by:

Baker et al.

If a CONTINUATION APPLICATION, check appropriate box and supply the requisite information:

☐ Continuation ☐ Divisional ☒ Continuation-in-part (CIP) of prior application No.: PCT/US99/13763

Which is a:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.:

Which is a:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.:

Enclosed are:

Application Elements

1. ☒ Filing fee as calculated and transmitted as described below
2. ☒ Specification having 70 pages and including the following:
 - a. ☒ Descriptive Title of the Invention
 - b. ☒ Cross References to Related Applications (if applicable)
 - c. ☐ Statement Regarding Federally-sponsored Research/Development (if applicable)
 - d. ☐ Reference to Microfiche Appendix (if applicable)
 - e. ☒ Background of the Invention
 - f. ☒ Brief Summary of the Invention
 - g. ☐ Brief Description of the Drawings (if drawings filed)
 - h. ☒ Detailed Description
 - i. ☒ Claim(s) as Classified Below
 - j. ☒ Abstract of the Disclosure

UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

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Application Elements (Continued)

3. ☐ Drawing(s) *(when necessary as prescribed by 35 USC 113)*
a. ☐ Formal b. ☐ Informal Number of Sheets _____
4. ☒ Oath or Declaration
a. ☐ Newly executed *(original or copy)* ☒ Unexecuted
b. ☐ Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional application only)*
c. ☒ With Power of Attorney ☐ Without Power of Attorney
d. ☐ DELETION OF INVENTOR(S)
Signed statement attached deleting inventor(s) named in the prior application,
see 37 C.F.R. 1.63(d)(2) and 1.33(b).
5. ☐ Incorporation By Reference *(usable if Box 4b is checked)*
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. ☐ Computer Program in Microfiche
7. ☒ Genetic Sequence Submission *(if applicable, all must be included)*
a. ☒ Paper Copy
b. ☒ Computer Readable Copy
c. ☒ Statement Verifying Identical Paper and Computer Readable Copy

Accompanying Application Parts

8. ☐ Assignment Papers *(cover sheet & documents)*
9. ☐ 37 CFR 3.73(b) Statement *(when there is an assignee)*
10. ☐ English Translation Document *(if applicable)*
11. ☐ Information Disclosure Statement/PTO-1449 ☐ Copies of IDS Citations
12. ☐ Preliminary Amendment
13. ☒ Acknowledgment postcard
14. ☒ Certificate of Mailing
☐ First Class ☒ Express Mail *(Specify Label No.):* **EL550127847US**

UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.
ISPH-0518

Total Pages in this Submission

Accompanying Application Parts (Continued)

15. ☐ Certified Copy of Priority Document(s) *(if foreign priority is claimed)*
16. ☒ Small Entity Statement(s) - Specify Number of Statements Submitted: 1
17. ☐ Additional Enclosures *(please identify below)*:

Request That Application Not Be Published Pursuant To 35 U.S.C. 122(b)(2)

18. ☐ Pursuant to 35 U.S.C. 122(b)(2), Applicant hereby requests that this patent application not be published pursuant to 35 U.S.C. 122(b)(1). Applicant hereby certifies that the invention disclosed in this application has not and will not be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication of applications 18 months after filing of the application.

Warning

An applicant who makes a request not to publish, but who subsequently files in a foreign country or under a multilateral international agreement specified in 35 U.S.C. 122(b)(2)(B)(i), must notify the Director of such filing not later than 45 days after the date of the filing of such foreign or international application. A failure of the applicant to provide such notice within the prescribed period shall result in the application being regarded as abandoned, unless it is shown to the satisfaction of the Director that the delay in submitting the notice was unintentional.

UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.
ISPH-0518

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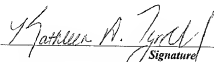
Fee Calculation and Transmittal

CLAIMS AS FILED

For	#Filed	#Allowed	#Extra	Rate	Fee
Total Claims	24	- 20 =	4	x \$9.00	\$36.00
Indep. Claims	1	- 3 =	0	x \$40.00	\$0.00
Multiple Dependent Claims (check if applicable) <input type="checkbox"/>					\$0.00
BASIC FEE					\$355.00
OTHER FEE (specify purpose)					\$0.00
TOTAL FILING FEE					\$391.00

- ☒ A check in the amount of **\$391.00** to cover the filing fee is enclosed.
- ☒ The Commissioner is hereby authorized to charge and credit Deposit Account No. **12-1086** as described below. A duplicate copy of this sheet is enclosed.
- ☐ Charge the amount of _____ as filing fee.
- ☒ Credit any overpayment.
- ☒ Charge any additional filing fees required under 37 C.F.R. 1.16 and 1.17.
- ☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b).

Dated: **October 24, 2000**


Signature

Kathleen A. Tyrrell
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cc:

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Page 1 of 2

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY
STATUS (37 CFR 1.9(f) AND 1.27 (e)) - SMALL BUSINESS CONCERN**

Docket No.
ISPT-0518

Serial No.

Not yet assigned.

Filing Date

Herewith

Patent No.

Issue Date

Applicant/ Baker et al.
Patentee:

Invention: Antisense Modulation of TNFR1 Expression

I hereby declare that I am:

- ☐ the owner of the small business concern identified below
☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: Isis Pharmaceuticals, Inc.

ADDRESS OF CONCERN: 2292 Faraday Avenue, Carlsbad, California 92008

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the above-identified invention described in:

- ☒ the specification filed herewith with title as listed above,
☐ the application identified above,
☐ the patent identified above.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed on the next page and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

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Page 2 of 2

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

- ☒ no such person, concern or organization exists.
☐ each such person, concern or organization is listed below.

FULL NAME

ADDRESS

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME

ADDRESS

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME

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☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME

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☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING:

B. Lynne Parrish

TITLE OF PERSON SIGNING

OTHER THAN OWNER:

Executive Vice President

ADDRESS OF PERSON SIGNING:

Isis Pharmaceuticals, Inc.Carlsbad Research Center2252 Faraday AvenueCarlsbad, CA 92008

SIGNATURE:

DATE: 10/23/2000

CERTIFICATE OF EXPRESS MAILING

"Express Mail" Label No.EL550127847US

Date of Deposit OCTOBER 24, 2000

I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R 1.10 on the date indicated above and is addressed to the "BOX SEQUENCE", Assistant Commissioner for Patents, Washington, D.C. 20231.

- 1) Patent Application Transmittal Letter (2 copies);
- 2) Application consisting of 70 pages of Specification, including three (3) pages of Claims, and one (1) page of Abstract;
- 3) Return Post Card;
- 4) Check in the amount of \$391.00;
- 5) Verified Statement Claiming Small Entity Status;
- 6) Unexecuted Declaration and Power of Attorney;
- 7) Statement to Support Filing and Submission in Accordance with 37 C.F.R. §§1.821-1.825;
- 8) Sequence listing; and
- 9) Diskette containing computer readable copy of Sequence Listing.

KATHLEEN A. TYRRELL

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

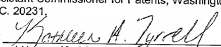
Attorney Docket No.: ISPH-0518
Inventors: Baker et al.
Serial No.: Not yet assigned.
Filing Date: Herewith
Examiner: Not yet assigned.
Group Art Unit: Not yet assigned.
Title: Antisense Modulation of TNFR1 Expression

"Express Mail" Label No. EL550127847US

Date of Deposit October 24, 2000

I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

By


Typed Name: Kathleen A. Tyrell

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Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

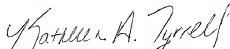
**STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE
WITH 37 CFR §§ 1.821 THROUGH 1.825**

- (XX) I hereby state, in accordance with the requirements of 37 C.F.R. §1.821(f), that the contents of the paper and computer readable copies of the Sequence Listing, submitted in accordance with 37 CFR §1.821(c) and (e), respectively are the same.
- () I hereby state that the submission filed in accordance with 37 CFR §1.821(g) does not include new matter.

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- () I hereby state that the submission filed in accordance with **37 CFR §1.821(h)** does not include new matter or go beyond the disclosure in the international application as filed.
- () I hereby state that the amendments, made in accordance with **37 CFR §1.825(a)**, included in the substitute sheet(s) of the Sequence Listing are supported in the application, as filed, at pages _____. I hereby state that the substitute sheet(s) of the Sequence Listing does not include new matter.
- () I hereby state that the substitute copy of the computer readable form, submitted in accordance with **37 CFR §1.825(b)**, is the same as the amended Sequence Listing.
- () I hereby state that the substitute copy of the computer readable form, submitted in accordance with **37 CFR §1.825(d)**, contains identical data to that originally filed.

Respectfully submitted,



Kathleen A. Tyrrell
Registration No. 38,350

Date: October 24, 2000

Law Offices of
JANE MASSEY LICATA
66 E. Main Street
Marlton, New Jersey 08053

(856) 810-1515

ANTISENSE MODULATION OF TNFR1 EXPRESSION

This application is a continuation-in-part of PCT/US99/13763 filed June 17, 1999 which claims priority to US Application Serial No. 09/106,038 filed June 26, 1998, now issued as U.S. Patent No. 6,007,995.

FIELD OF THE INVENTION

The present invention provides compositions and methods of modulating the expression of TNFR1. In particular, this invention relates to antisense compounds, particularly
10 oligonucleotides, specifically hybridizable with nucleic acids encoding human TNFR1. Such oligonucleotides have been shown to modulate the expression of TNFR1.

BACKGROUND OF THE INVENTION

One of the principal mechanisms by which cellular
15 regulation is effected is through the transduction of extracellular signals into intracellular signals that in turn modulate biochemical pathways. Examples of such extracellular signaling molecules include growth factors, cytokines, and chemokines. The cell surface receptors of these molecules and
20 their associated signal transduction pathways are therefore one of the principal means by which cellular behavior is regulated. Because cellular phenotypes are largely influenced by the activity of these pathways, it is currently believed that a number of disease states and/or disorders are a result
25 of either aberrant activation or functional mutations in the molecular components of signal transduction pathways.

For example, the polypeptide cytokine tumor necrosis factor (TNF) is normally produced during infection, injury, or invasion where it serves as a pivotal mediator of the
30 inflammatory response. In recent years, a number of in vivo

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animal and human studies have demonstrated that overexpression of TNF by the host in response to disease and infection is itself responsible for the pathological consequences associated with the underlying disease. For example, septic shock as a result of massive bacterial infection has been attributed to infection-induced expression of TNF. Thus, systemic exposure to TNF at levels comparable to those following massive bacterial infection has been shown to result in a spectrum of symptoms (shock, tissue injury, capillary leakage, hypoxia, pulmonary edema, multiple organ failure, and high mortality rate) that is virtually indistinguishable from septic shock syndrome. Tracey, 1994, Ann. Rev. Med. 45, 491-503. Further evidence has been provided in animal models of septic shock, in which it has been demonstrated that systemic exposure to anti-TNF neutralizing antibodies block bacterial-induced sepsis. Tracey, 1994, Ann. Rev. Med. 45, 491-503. In addition to these acute effects, chronic exposure to low-dose TNF, results in a syndrome of cachexia marked by anorexia, weight loss, dehydration, and depletion of whole-body protein and lipid. Chronic production of TNF has been implicated in a number of diseases including AIDS and cancer. Tracey, 1994, Ann. Rev. Med. 45, 491-503. To date, two distinct TNF cells surface receptors, known as TNFR1 and TNFR2, have been described. Molecular analysis of TNFR1 and TNFR2 have shown that the two receptors share little homology in their intracellular domains and appear to activate distinct intracellular pathways. Tracey, 1994, Ann. Rev. Med. 45, 491-503.

Recent studies with transgenic TNFR1 knockout mice have demonstrated that signalling through TNFR1 plays an important role in the clearing of low-level bacterial infection as well as TNF-induced septic shock following high-level bacterial infection. Lotz, 1996, J. Leukoc. Biol., 60, 1-7. These findings indicate that compositions of matter which can

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inhibit signalling through the TNFR1 receptor may serve as useful targets for inhibition of TNF induced toxicities such as septic shock.

Antisense oligonucleotide inhibition of TNFR1 has proven to be a useful tool in understanding the role of TNFR1 stimulation in cytokine induction and cell proliferation. Ojwang et. al. have disclosed partial phosphothioate antisense oligodeoxynucleotides containing C-5 propynyl or hexynyl derivatives of 2'-deoxyuridine which caused attenuation of TNFR1 mRNA and protein and inhibited TNF-alpha induced expression of IL-6 in MRC-5 cells. Ojwang, 1997, Biochemistry, 36, 6033-6045. These oligonucleotides were targeted to the poly (A) signal site of TNFR1 mRNA. A uniform phosphorothioate oligonucleotide targeted to the translation initiation codon of TNFR1 was found to have no effect.

There remains a long-felt need for improved compositions and methods for inhibiting TNFR1 gene expression.

SUMMARY OF THE INVENTION

The present invention is directed to antisense compounds, particularly oligonucleotides, which are targeted to a nucleic acid encoding TNFR1, and which modulate the expression of TNFR1. Pharmaceutical and other compositions comprising the antisense compounds of the invention are also provided. Further provided are methods of modulating the expression of TNFR1 in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of TNFR1 by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

5 The present invention employs oligomeric antisense compounds, particularly oligonucleotides, for use in modulating the function of nucleic acid molecules encoding

10 TNFR1, ultimately modulating the amount of TNFR1 produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding TNFR1. As used herein, the terms "target nucleic acid" and "nucleic acid encoding TNFR1" encompass DNA encoding TNFR1,

15 RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic

20 acid by compounds which specifically hybridize to it is generally referred to as "antisense." The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation

25 of the expression of TNFR1. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred

30 target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification

35 of a nucleic acid sequence whose function is to be modulated.

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This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, 5 the target is a nucleic acid molecule encoding TNFR1. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context 10 of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the 15 corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon." A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to 20 function in vivo. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (prokaryotes). It is also known in the art that eukaryotic and 25 prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation 30 codon" refer to the codon or codons that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding TNFR1, regardless of the sequence(s) of such codons.

It is also known in the art that a translation 35 termination codon (or "stop codon") of a gene may have one of

three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of 5 such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses 10 from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the 15 translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, 20 and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene) and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, 25 and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene). The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap 30 region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly 35 translated, many contain one or more regions, known as

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"introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon
5 junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions
10 are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified,
15 oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or
20 reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two
25 nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position.
30 The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to
35 indicate a sufficient degree of complementarity or precise

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pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target
5 nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity
10 to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, or in the case of in vitro assays, under conditions in which the assays
15 are performed.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of
20 ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

25 The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotides have been safely and
30 effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

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In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 30 nucleobases. Particularly preferred are antisense oligonucleotides comprising from about 8 to about 30 nucleobases (i.e. from about 8 to about 30 linked nucleosides). As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure,

the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

5 Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom
10 in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Patents: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,625,050; and 5,697,248.

certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are
5 formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from
10 the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino
15 backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not
20 limited to, U.S. Patents: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070;
25 5,663,312; 5,633,360; 5,677,437; and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of
30 the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a
35 peptide nucleic acid (PNA). In PNA compounds, the sugar-

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backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Patents: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, 1991, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-\text{CH}_2-\text{NH}-\text{O}-\text{CH}_2-$, $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{O}-\text{CH}_2-$ [known as a methylene (methylimino) or MMI backbone], $-\text{CH}_2-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-$, $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{N}(\text{CH}_3)-\text{CH}_2-$ and $-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-$ [wherein the native phosphodiester backbone is represented as $-\text{O}-\text{P}-\text{O}-\text{CH}_2-$] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. Patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl, O-, S-, or N-alkenyl, O, S- or N-alkynyl, or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $\text{O}[(\text{CH}_2)_n\text{O}]_m\text{CH}_3$, $\text{O}(\text{CH}_2)_n\text{OCH}_3$, $\text{O}(\text{CH}_2)_n\text{NH}_2$, $\text{O}(\text{CH}_2)_n\text{CH}_3$, $\text{O}(\text{CH}_2)_n\text{ONH}_2$, and $\text{O}(\text{CH}_2)_n\text{ON}[(\text{CH}_2)_m\text{CH}_3]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3 , OCN, Cl, Br, CN, CF_3 , OCF_3 , SOCH_3 , SO_2CH_3 , ONO_2 , NO_2 , N_3 , NH_2 , heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, poly-

alkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoxyethoxy, i.e., a O(CH₂)₂ON(CH₃)₂ group, also known as 2'-DMAOE, as described in United States patent application Serial Number 09/016,520, filed on January 30, 1998, which is commonly owned with the instant application and the contents of which are herein incorporated by reference.

Other preferred modifications include 2'-methoxy (2'-O-CH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugars structures include, but are not limited to, U.S. Patents: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and U.S. Patent 5,859,221, which is commonly owned with the instant application and is also herein incorporated by reference.

Oligonucleotides may also include nucleobase (often

DISCUSSION

Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

5 Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Patent 3,687,808, as well as U.S. Patents: 4,845,205; 5,130,302; 5,134,066; 5,175,273;
10 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and U.S. Patent
15 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity,
20 cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 1989, 86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4,
25 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660, 306-309; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl
30 residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10, 1111-1118; Kabanov et al., *FEBS Lett.*, 1990, 259, 327-330; Svinarchuk et al., *Biochimie*, 1993, 75, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate

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(Manoharan et al., *Tetrahedron Lett.*, **1995**, 36, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, **1995**, 14, 969-973), or adamantane

5 acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, 36, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, **1995**, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxysterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, **1996**, 277, 923-937).

10 Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. Patents: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124;

15 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469;

20 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned

25 with the instant application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a

30 single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly

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oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region

5 wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for

10 enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide

15 inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely

20 detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides

25 and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S. Patents: 5,013,830; 5,149,797; 5,220,007;

30 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and U.S. Patent 5,955,589 which is commonly owned with the instant

35 application and also herein incorporated by reference.

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The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the in vivo synthesis of antisense molecules.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S. Patents: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also

drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that
5 is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE
10 [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the
15 compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline
20 earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chlorprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine,
25 N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, **1977**, *66*, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the
30 conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but
35 otherwise the salts are equivalent to their respective free

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acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or
5 inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as,
10 for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid,
15 maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic
20 acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid,
25 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates),
30 or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth,

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ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine. The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of TNFR1 is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding TNFR1, enabling sandwich and other assays to easily be constructed to exploit this fact.

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Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding TNFR1 can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits 5 using such detection means for detecting the level of TNFR1 in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

25 Pharmaceutical compositions and formulations for topical
administration may include transdermal patches, ointments,
lotions, creams, gels, drops, suppositories, sprays, liquids
and powders. Conventional pharmaceutical carriers, aqueous,
powder or oily bases, thickeners and the like may be necessary
30 or desirable. Coated condoms, gloves and the like may also
be useful.

Compositions and formulations for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets.

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Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include
5 sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions and/or formulations
10 comprising the oligonucleotides of the present invention may also include penetration enhancers in order to enhance the alimentary delivery of the oligonucleotides. Penetration enhancers may be classified as belonging to one of five broad categories, i.e., fatty acids, bile salts, chelating agents,
15 surfactants and non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, 8, 91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33). One or more penetration enhancers from one or more of these broad categories may be included.
20 Penetration enhancers are described in pending United States patent application 08/886,829, filed on July 1, 1997, and U.S. Patent 6,083,923 both of which are commonly owned with the instant application and both of which are herein incorporated by reference.

25 Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, recinleate, monoolein (a.k.a. 1-monooleoyl-rac-glycerol),
30 dilaurin, caprylic acid, arichidonic acid, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, mono- and di-glycerides and physiologically acceptable salts thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al.,

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Critical Reviews in Therapeutic Drug Carrier Systems, 1991, 8:2, 91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7:1, 1-33; El-Hariri et al., *J. Pharm. Pharmacol.*, 1992, 44, 651-654). Examples of some presently preferred fatty acids are sodium caprate and sodium laurate, used singly or in combination at concentrations of 0.5 to 5%.

Preferred penetration enhancers are disclosed in pending United States patent application 08/886,829, filed on July 1, 1997, which is commonly owned with the instant application and which is herein incorporated by reference.

The physiological roles of bile include the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, NY, 1996, pages 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salt" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. Preferred bile salts are described in pending United States patent application 08/886,829, filed on July 1, 1997, which is commonly owned with the instant application and which is herein incorporated by reference. A presently preferred bile salt is chenodeoxycholic acid (CDCA) (Sigma Chemical Company, St. Louis, MO), generally used at concentrations of 0.5 to 2%.

Complex formulations comprising one or more penetration enhancers may be used. For example, bile salts may be used in combination with fatty acids to make complex formulations. Preferred combinations include CDCA combined with sodium caprate or sodium laurate (generally 0.5 to 5%).

Chelating agents include, but are not limited to, disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and

N-amino acyl derivatives of beta-diketones (enamines) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, 8:2, 92-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7:1, 1-33; Buur et al., *J. Control Rel.*, 1990, 14, 43-51). Chelating agents have the added advantage of also serving as DNase inhibitors.

Surfactants include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, 8:2, 92-191); and perfluorochemical emulsions, such as FC-43 (Takahashi et al., *J. Pharm. Pharmacol.*, 1988, 40, 252-257).

Non-surfactants include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, 8:2, 92-191); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., *J. Pharm. Pharmacol.*, 1987, 39, 621-626).

As used herein, "carrier compound" refers to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity per se) but is recognized as a nucleic acid by in vivo processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioated oligonucleotide in hepatic tissue is reduced when it is

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coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (Miyao et al., *Antisense Res. Dev.*, **1995**, *5*, 115-121; Takakura et al., *Antisense & Nucl. Acid Drug Dev.*, **1996**, *6*, 177-183).

In contrast to a carrier compound, a "pharmaceutically acceptable carrier" (excipient) is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The pharmaceutically acceptable carrier may be liquid or solid and is selected with the planned manner of administration in mind so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutically acceptable carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrates (e.g., starch, sodium starch glycolate, etc.); or wetting agents (e.g., sodium lauryl sulphate, etc.). Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are described in U.S. Patents: 4,704,295; 4,556,552; 4,309,406; and 4,309,404.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain 35 additional compatible pharmaceutically-active materials such

as, e.g., antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the composition of present invention, such as dyes, flavoring
5 agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the invention.

Regardless of the method by which the antisense
10 compounds of the invention are introduced into a patient, colloidal dispersion systems may be used as delivery vehicles to enhance the in vivo stability of the compounds and/or to target the compounds to a particular organ, tissue or cell type. Colloidal dispersion systems include, but are not
15 limited to, macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, liposomes and lipid:oligonucleotide complexes of uncharacterized structure. A preferred colloidal dispersion system is a plurality of
20 liposomes. Liposomes are microscopic spheres having an aqueous core surrounded by one or more outer layer(s) made up of lipids arranged in a bilayer configuration (see, generally, Chonn et al., *Current Op. Biotech.*, 1995, 6, 698-708).

Liposome preparation is described in U.S. Patent 6,083,923,
25 which is commonly owned with the instant application and which is herein incorporated by reference.

Certain embodiments of the invention provide for liposomes and other compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic
30 agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include, but are not limited to, anticancer drugs such as daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine,
35 6-thioguanine, cytarabine (CA), 5-fluorouracil (5-FU),

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floxuridine (5-FuDR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, 5 Rahway, N.J., pages 1206-1228). Antiinflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the 10 invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together 15 or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Examples of antisense oligonucleotides include, but are not limited to, those directed to the following targets as disclosed in the indicated U.S. Patents, or pending U.S. applications, which are commonly owned with the instant application and are hereby incorporated by reference, or the indicated published PCT applications: raf (WO 96/39415, WO 95/32987 and U.S. Patent 5,563,255, issued October 8, 1996, and 5,656,612, issued August 12, 1997), the p120 nucleolar antigen (WO 93/17125 and U.S. Patent No. 5,656,743, issued August 12, 1997), protein kinase C (WO 95/02069, WO 95/03833 and WO 93/19203), multidrug resistance-associated protein (WO 95/10938 and U.S. Patent 5,510,239, issued March 23, 1996), subunits of transcription factor AP-1 (pending application U.S. Serial No. 08/837,201, filed April 14, 1997), Jun kinases (pending application U.S. Serial No. 08/910,629, filed August 13, 1997), MDR-1

(multidrug resistance glycoprotein; pending application U.S. Serial No. 08/731,199, filed September 30, 1997), HIV (U.S. Patents 5,166,195, issued November 24, 1992 and 5,591,600, issued January 7, 1997), herpesvirus (U.S. Patent 5,248,670, issued September 28, 1993 and U.S. Patent 5,514,577, issued May 7, 1996), cytomegalovirus (U.S. Patents 5,442,049, issued August 15, 1995 and 5,591,720, issued January 7, 1997), papillomavirus (U.S. Patent 5,457,189, issued October 10, 1995), intercellular adhesion molecule-1 (ICAM-1) (U.S. Patent 5,514,788, issued May 7, 1996), 5-lipoxygenase (U.S. Patent 5,530,114, issued June 25, 1996) and influenza virus (U.S. Patent 5,580,767, issued December 3, 1996). Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC₅₀s found to be effective in in vitro and in vivo animal models. In general, dosage is from 0.01 µg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the

oligonucleotide is administered in maintenance doses, ranging from 0.01 :g to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with
5 specificity in accordance with certain of its preferred
embodiments, the following examples serve only to illustrate
the invention and are not intended to limit the same.

EXAMPLES

Example 1

10 Nucleoside Phosphoramidites for Oligonucleotide Synthesis
Deoxy and 2'-alkoxy amidites

2'-Deoxy and 2'-methoxy beta-cyanoethyl diisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids Research*, **1993**, *21*, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

2'-Fluoro amidites

2'-Fluorodeoxvadenosine amidites

2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, 1993, 36, 831-841] and U.S. Patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-

deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S_N2 -5 displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard 10 methods were used to obtain the 5'-dimethoxytrityl- (DMT) and 5'-DMT-3'-phosphoramidite intermediates.

2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropylidisiloxanyl (TPDS) protected 15 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutryl di-THP protected arabinofuranosylguanine. 20 Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

25 Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'-phosphoramidites.

30 2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection

to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'-phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

- 5 2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, 1995, 78, 486-504.

10 **2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]**

- 5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated
15 to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether
20 was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57
25 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white
30 solid, mp 222-4°C).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH₃CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH₂Cl₂/acetone/MeOH (20:5:3) containing 0.5% Et₃NH. The residue was dissolved in CH₂Cl₂ (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH₃CN (200 mL). The residue was dissolved in CHCl₃ (1.5 L) and extracted with 2x500 mL of saturated NaHCO₃ and 2x500 mL of saturated NaCl. The organic phase was dried over Na₂SO₄, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/Hexane/Acetone (5:5:1) containing 0.5% Et₃NH. The pure

fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in CHCl_3 (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of CHCl_3 . The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/Hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. POCl_3 was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The

first solution was added dropwise, over a 45 minute period, to the later solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue
5 was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO₃ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

10 **2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine**

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH₄OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated
15 and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH₃ gas was added and the vessel heated to 100°C for 2 hours (tlc showed complete conversion). The vessel contents were
20 evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

25 **N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine**

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, tlc showed the reaction to be
30 approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl₃ (700 mL) and extracted with saturated NaHCO₃ (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO₄ and

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evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (1:1) containing 0.5% Et₃NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the
5 title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH₂Cl₂ (1 L). 10 Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction mixture was 15 extracted with saturated NaHCO₃ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH₂Cl₂ (300 mL), and the extracts were combined, dried over MgSO₄ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (3:1) as the eluting 20 solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

2'-(Aminooxyethyl) nucleoside amidites and 2'-(dimethylamino-oxyethyl) nucleoside amidites

Aminooxyethyl and dimethylaminooxyethyl amidites are
25 prepared as per the methods of U.S. Patent applications serial
numbers 10/037,143, filed February 14, 1998, and 09/016,520,
filed January 30, 1998, each of which is commonly owned with
the instant application and is herein incorporated by
reference.

Example 2

Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 hr), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as
20 described in U.S. Patent 4,469,863, herein incorporated by
reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

25 Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and 30 PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 4

PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, 1996, 4, 5-23.

They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

Example 5

5 Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between
10 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of
15 the second type are also known in the art as "hemimers" or "wingmers."

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl
20 phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and
25 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from
30 the support and the phosphate group is deprotected in 3:1 Ammonia/Ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect all bases and

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sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by 5 rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometer.

10 [2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate
 Oligonucleotides

 [2'-O-(2-methoxyethyl)]--[2'-deoxy]--[2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl
15 chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

 [2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy
Phosphorothioate]--[2'-O-(2-Methoxyethyl)
Phosphodiester] Chimeric Oligonucleotides

20 [2'-O-(2-methoxyethyl phosphodiester)]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites,
25 oxidization with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center
30 gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are

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synthesized according to U.S. Patent 5,623,065, herein incorporated by reference.

Example 6

Oligonucleotide Isolation

- 5 After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed
- 10 by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ³¹P nuclear magnetic resonance spectroscopy, and for some studies
- 15 oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* **1991**, *266*, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 7

20 Oligonucleotide Synthesis - 96 Well Plate Format

- Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were
- 25 afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,4,5-benzodithiole-3-one 1,1-dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyl-diisopropyl phosphoramidites were purchased
- 30 from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or

patented methods. They are utilized as base protected beta-cyanoethyl-diisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature 5 (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

10 **Example 8**

Oligonucleotide Analysis - 96 Well Plate Format

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual 15 products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds 20 utilizing Electrospray-Mass Spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

25 **Example 9**

Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable 30 levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following four cell types

are provided for illustrative purposes, but other cell types can be routinely used.

T-24 cells:

The transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

20 A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 25 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

30 NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville MD). NHDFs were

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routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

5 HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as recommended by the supplier. Cell were routinely maintained for up to 10 passages as recommended by the supplier.

b.END cells:

The mouse brain endothelial cell line b.END was obtained from Dr. Werner Risau at the Max Plank Institute (Bad Nauheim, Germany). b.END cells were routinely cultured in DMEM, high glucose (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon Primaria #3872) at a density of 3000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and 25 treated similarly, using appropriate volumes of medium and oligonucleotide.

Treatment with antisense compounds:

When cells reached 80% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells 30 were washed once with 200 μ L OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130 μ L of OPTI-MEM™-1 containing 3.75 μ g/mL LIPOFECTIN™ (Gibco BRL) and the desired

oligonucleotide at a final concentration of 150 nM. After 4 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16 hours after oligonucleotide treatment.

Example 10

5 Antisense inhibition of TNFR1 expression- phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human TNFR1 RNA, using published sequences (GenBank
10 accession number X55313, incorporated herein as SEQ ID NO: 1). The oligonucleotides are shown in Table 1. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. X55313), to which the oligonucleotide binds. All compounds in Table 1 are
15 oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. The compounds were analyzed for effect on TNFR1 mRNA levels by quantitative real-time PCR as described in subsequent examples. Data are averages from three experiments.

20

Table 1

Inhibition of TNFR1 mRNA levels by phosphorothioate
oligodeoxynucleotides

	ISIS#	REGION	TARGET	SEQUENCE	%	SEQ ID
			SITE		Inhibition	NO.
	18875	5' UTR	37	TTCTCTGGACTGAGGCTC	19	8
25	18876	5' UTR	68	TCCCTCTCTCTCTGCTTT	5	9
	18877	5' UTR	109	AGACTCGGGCATAGAGAT	0	10
	18878	5' UTR	114	GGTTGAGACTCGGGCATA	40	11
	18879	5' UTR	118	TGAGGGTTGAGACTCGGG	2	12
	18880	5' UTR	123	ACAGTTGAGGGTTGAGAC	30	13
30	18881	5' UTR	127	GGTGACAGTTGAGGGTTG	8	14
	18882	5' UTR	196	GCAGTGTGGCAGCGCAG	53	15
	18883	5' UTR	199	AGGGCAGTGTGGCAGCGG	53	16

	18884	5' UTR	202	CTCAGGGCAGTGTGGCAG	61	17
	18885	5' UTR	207	TTGGGCTCAGGGCAGTGT	0	18
	18886	5' UTR	210	CATTTGGGCTCAGGGCAG	9	19
	18887	Coding	262	GTCAGGCACGGTGGAGAG	0	20
5	18888	Coding	266	GCAGGTCAGGCACGGTGG	16	21
	18889	Coding	272	GCAGCAGCAGGTCAGGCA	37	22
	18890	Coding	276	AGCGGCAGCAGCAGGTCA	0	23
	18891	Coding	280	CACGAGCGGCAGCAGCAG	21	24
	18892	Coding	286	CAGGAGCACGAGCGGAG	46	25
10	18893	Coding	306	TATATTTCCACCAACAGC	25	26
	18894	Coding	356	TCTTCTCCCTGTCCCTTA	13	27
	18895	Coding	403	ATTATTTTGAGGTGGAT	0	28
	18896	Coding	435	GTTCTTTTGTGGCACTTG	12	29
	18897	Coding	440	AGTAGGTTCTTTTGTGGC	46	30
15	18898	Coding	460	GCCTGGACAGTCATTGTA	0	31
	18899	Coding	480	CAGTCCGTATCCTGCCCC	26	32
	18900	Coding	500	AGCCGCTCTCACTCTCC	36	33
	18901	Coding	516	TCTGAAGCGGTGAAGGAG	0	34
	18902	Coding	521	GGTTTCTGAAGCGGTGA	17	35
20	18903	Coding	525	AGGTGGTTTCTGAAGCG	0	36
	18904	Coding	530	GTCTGAGGTGGTTTCTG	34	37
	18905	Coding	537	AGGCAGTGTCTGAGGTGG	0	38
	18906	Coding	542	AGCTGAGGCAGTGTCTGA	27	39
	18907	Coding	565	CATTTCTTTTCGGCATTT	13	40
25	18908	Coding	569	GACCCATTTCCTTTTCGGC	26	41
	18909	Coding	574	CACCTGACCCATTTCCTT	46	42
	18910	Coding	635	GGTACTGGTTCTTCCTGC	14	43
	18911	Coding	654	TTTTCACTCCAATAATGC	0	44
	18912	Coding	693	CCATTGAGGCAGAGGCTG	48	45
30	18913	Coding	699	ACGGTCCCATTTGAGGCAG	34	46
	18914	Coding	732	ACGGTGTCTGTCTCTCC	7	47
	18915	Coding	786	CTACAGGAGACACACTCG	28	48
	18916	Coding	796	CTTACAGTTACTACAGGA	21	49
	18917	Coding	802	GCTTTTCTTACAGTTACT	10	50
35	18918	Coding	807	TCCAGGCTTTTCTTACAG	0	51
	18919	Coding	845	TAACATTCTCAATCTGGG	0	52
	18920	Coding	873	ACTGTGGTGCCCTGAGTCC	31	53
	18921	Coding	906	CAAAGACCAAGAAAATG	29	54
	18922	Coding	911	AAAGGCAAAGACCAAGA	31	55
40	18923	Coding	921	AGGAGGGATAAAAGGCAA	22	56
	18924	Coding	929	CAATGAAGAGGAGGGATA	21	57
	18925	Coding	935	TTAAACCAATGAAGAGGA	28	58
	18926	Coding	952	CCGTTGGTAGCGATACAT	30	59

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	18927	Coding	992	TCGATTTCCCAACAA	1	60
	18928	Coding	1033	CTTAGTAGTAGTTCCTTC	15	61
	18929	Coding	1075	GAAGCCTGGAGTGGGACT	48	62
	18930	Coding	1098	GGACTGAAGCCCAGGGTG	12	63
5	18931	Coding	1113	GTGGAACCTGGGCACGGGA	4	64
	18932	Coding	1118	TGAAGGTGGAACCTGGGCA	27	65
	18933	Coding	1127	AGCTGGAGGTGAAGGTGG	0	66
	18934	Coding	1162	CGCAAAGTTGGGACAGTC	30	67
	18935	Coding	1184	GTGCCACCTCTCTGCGGG	0	68
10	18936	Coding	1269	CTGTCTCCCACTTCTGA	16	69
	18937	Coding	1290	AGGCTCTGTGGCTTGTGG	47	70
	18938	Coding	1389	TCGTGGTCGCTCAGCCCT	28	71
	18939	Coding	1465	CCGCCTCCAGTGCAGCAG	0	72
	18940	Coding	1537	GCAGCCCAGCAGGTCCAT	32	73
15	18941	Coding	1545	TCCTCCAGGCAGCCCAGC	41	74
	18942	Coding	1604	ATCTGAGAAGACTGGGCG	0	75
	18943	Coding	1707	GCTCCTGCTTGCCCCCTGC	43	76
	18944	Coding	1732	GTTAGCACCAAGTAGGCG	11	77
	18945	Coding	1842	CGCAAACCACCCACTCAG	51	78
20	18946	Coding	1847	ATCCTCGCAAACACCCCA	29	79
	18947	Coding	1859	ATAGCGTCCCTCATCTC	34	80
	18948	Coding	1925	CTCAGGGACGAACACAGGG	3	81
	18949	Coding	1930	AAAGGCTCAGGGACGAAC	42	82
	18950	Coding	1979	ACAAAACAAAACAAAACA	27	83
25	18951	Coding	2016	GCCAAGTTTCTATTAGTG	10	84
	18952	Coding	2033	GCAGAGGGCACAGGAGTG	24	85
	18953	Coding	2039	GTCCAGGCAGAGGGCACA	21	86
	18954	Coding	2043	GCTTGTCCAGGCAGAGGG	18	87
	18955	Coding	2071	TGCCTTAGGACAGTTTCAG	20	88
30	18956	Coding	2085	TCCGTGCTCGCCCCCTGCC	19	89
	18957	Coding	2089	TGTTCCTGTGCTCGCCCC	41	90
	18958	Coding	2097	AGGCCCCATTGTTCCGTG	0	91

As shown in Table 1, SEQ ID NOs 11, 15, 16, 17, 22, 25, 30, 33, 42, 45, 62, 70, 74, 76, 78, 82 and 90 demonstrated at least 35% inhibition of TNFR1 expression in this assay and are therefore preferred.

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Example 11**Analysis of oligonucleotide inhibition of TNFR1 expression**

Antisense modulation of TNFR1 expression can be assayed in a variety of ways known in the art. For example, TNFR1 mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., 1996. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions. Other methods of PCR are also known in the art.

TNFR1 protein levels can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to TNFR1 can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., 1997. Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., 1997.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., 1998. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., 1997. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., 1991.

Example 12

Poly(A) + mRNA isolation

Poly(A)+ mRNA was isolated according to Miura et al.,
15 Clin. Chem., 1996, 42, 1758-1764. Other methods for poly(A)+
mRNA isolation are taught in, for example, Ausubel, F.M. et
al., Current Protocols in Molecular Biology, Volume 1, pp
4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Briefly, for cells
grown on 96-well plates, growth medium was removed from the
20 cells and each well was washed with 200 μ L cold PBS. 60 μ L
lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl,
0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added
to each well, the plate was gently agitated and then incubated
at room temperature for five minutes. 55 μ L of lysate was
25 transferred to Oligo d(T) coated 96-well plates (AGCT Inc.,
Irvine CA). Plates were incubated for 60 minutes at room
temperature, washed 3 times with 200 μ L of wash buffer (10 mM
Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash,
the plate was blotted on paper towels to remove excess wash
30 buffer and then air-dried for 5 minutes. 60 μ L of elution
buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to
each well, the plate was incubated on a 90° hot plate for 5

minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

5 Example 13

Total RNA Isolation

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Total mRNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μ L cold PBS. 100 μ L Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 μ L of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 μ L water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 μ L water.

Example 14**Real-time Quantitative PCR Analysis of TNFR1 mRNA Levels**

Quantitation of TNFR1 mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence
5 Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard
10 PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR
15 primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE or FAM, PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact,
20 reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of
25 the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is
30 monitored at regular (six-second) intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent

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inhibition after antisense oligonucleotide treatment of test samples.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding
5 25 μ L PCR cocktail (1x TAQMAN[™] buffer A, 5.5 mM $MgCl_2$, 300 μ M each of dATP, dCTP and dGTP, 600 μ M of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNase inhibitor, 1.25 Units AMPLITAQ GOLD[™], and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25 μ L
10 poly(A) mRNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD[™], 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes
15 (annealing/extension).

For TNFR1 the PCR primers were:

forward primer: GCTTCAGAAAACACCTCAGACA (SEQ ID No. 2)

reverse primer: CCGGTCCACTGTGCAAGAA (SEQ ID No. 3) and the PCR

probe was: FAM-TCAGCTGCTCCAAATGCCGAAAGG-TAMRA

20 (SEQ ID No. 4) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

For GAPDH the PCR primers were:

forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID No. 5)

25 reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID No. 6) and the PCR

probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC- TAMRA 3' (SEQ ID No.

7) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

30 Example 15

Northern blot analysis of TNFR1 mRNA levels

Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL RNAZOL[™] (TEL-TEST "B" Inc., Friendswood, TX). Total RNA was prepared

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following manufacturer's recommended protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon, OH). RNA was transferred from the gel to HYBOND™-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc, La Jolla, CA).

Membranes were probed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions with a TNFR1 specific probe prepared by PCR using the forward primer GCTTCAGAAAACCCACCTCAGACA (SEQ ID No. 2) and the reverse primer CCGGTCCACTGTGCAAGAA (SEQ ID No. 3). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) RNA (Clontech, Palo Alto, CA). Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to G3PDH levels in untreated controls.

Example 16

Western blot analysis of TNFR1 protein levels

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 hr after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100μl/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to TNFR1 is used, with a radiolabelled or fluorescently labeled secondary antibody

directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

Example 17:

5 Antisense inhibition of TNFR1 expression- phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a second series of oligonucleotides targeted to human TNFR1 were synthesized. The oligonucleotide sequences are shown in Table 2. Target
10 sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. X55313), to which the oligonucleotide binds.

All compounds in Table 2 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central
15 "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings." The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide.
20 Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as described in previous examples and are averaged from three experiments.

Table 2

25 Inhibition of TNFR1 mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS#	REGION	TARGET	SEQUENCE	%	SEQ ID
			SITE	Inhibition	NO.
19463	5' UTR	37	TTCTCTGGACTGAGGCTC	72	8
30 19464	5' UTR	68	TCCCCCTCCTCTCTGCTTT	9	9
19465	5' UTR	109	AGACTCGGGCATAGAGAT	18	10

5	19466	5' UTR	114	GGTTGAGACTCGGGCATA	95	11
	19467	5' UTR	118	TGAGGGTTTGAGACTCGGG	28	12
	19468	5' UTR	123	ACAGTTGAGGGTTTGAGAC	66	13
	19469	5' UTR	127	TGTGACAGTTGAGGGTTTG	42	14
	19470	5' UTR	196	GCAGTGTGGCAGCGGCAG	78	15
10	19471	5' UTR	199	AGGGCAGTGTGGCAGCGG	76	16
	19472	5' UTR	202	CTCAGGGCAGGTGTGGCAG	90	17
	19473	5' UTR	207	TTGGGCTCAGGCAGTGT	48	18
	19474	5' UTR	210	CATTTGGGCTCAGGGCAG	70	19
	19475	Coding	262	GTCAGGCACGGTGAGAG	66	20
15	19476	Coding	266	GCAGGTCAGGCACGGTGG	91	21
	19477	Coding	272	GCAGCAGCAGGTGAGGCA	85	22
	19478	Coding	276	AGCGGCAGCAGCAGGTCA	93	23
	19479	Coding	280	CACCAGCGCGCAGCAGCAG	65	24
	19480	Coding	286	CAGGAGCACCAGCGGCAG	60	25
20	19481	Coding	306	TATATTCCCAACACAGC	58	26
	19482	Coding	356	TCTTCTCCTGTCCCCTA	42	27
	19483	Coding	403	ATTATTTTGAGGGTGGAT	75	28
	19484	Coding	435	GTTCTTTTGTGGCACTTG	88	29
	19485	Coding	440	AGTAGGTTCTTTGTGGC	78	30
25	19486	Coding	460	GCCTGGACAGTCATTGTA	80	31
	19487	Coding	480	CAGTCCGATCTCTGCCCC	66	32
	19488	Coding	500	AGCCGCTCTCACACTCCC	86	33
	19489	Coding	516	TCTGAAGCGCTGAAGGAG	52	34
	19490	Coding	521	GGTTTTCTGAAGCGGTGA	92	35
30	19491	Coding	525	AGGTGGTTTTCTGAAGCG	82	36
	19492	Coding	530	GTCTGAGGTGGTTTCTGT	91	37
	19493	Coding	537	AGGCAGTGTCTGAGGTGG	96	38
	19494	Coding	542	AGCTGAGGCAGTGTCTGA	79	39
	19495	Coding	565	CATTTTCCTTCGGCATTT	41	40
35	19496	Coding	569	GACCCATTTCTTTTCGGC	93	41
	19497	Coding	574	CACCTGACCCATTTCTCTT	63	42
	19498	Coding	635	GGTACTGGTCTCTCTCTGC	79	43
	19499	Coding	654	TTTTCCTACTCCAAATATGC	9	44
	19500	Coding	693	CCATTGAGCAGAGGCTGT	0	45
40	19501	Coding	699	ACGGTCCCATTGAGGCAG	81	46
	19502	Coding	732	ACGGTGTTCTGTCTCTCC	77	47
	19503	Coding	786	CTACAGGAGACCACTCG	81	48
	19504	Coding	796	CTTACAGTTTACTACAGGA	61	49
	19505	Coding	802	GCTTTTCTTACAGTTACT	93	50
45	19506	Coding	807	TCCAGGCTTTTCTTACAG	71	51
	19507	Coding	845	TAACATTCTCACTGTGGG	0	52
	19508	Coding	873	ACTGTGGTGCTCTAGTCC	74	53

	19509	Coding	906	CAAAGACCAAAGAAAATG	29	54
	19510	Coding	911	AAAGGCAAAGACCAAAGA	45	55
	19511	Coding	921	AGGAGGGATAAAAGGCAA	67	56
	19512	Coding	929	CAATGAAGAGGAGGGATA	55	57
5	19513	Coding	935	TTAAACCAATGAAGAGGA	25	58
	19514	Coding	952	CCGTGGTAGCGATACAT	93	59
	19515	Coding	992	TCGATTTCACAAACAA	16	60
	19516	Coding	1033	CTTAGTAGTAGTTCCTTC	70	61
	19517	Coding	1075	GAAGCCTGGAGTGGGACT	0	62
10	19518	Coding	1098	GGACTGAAGCCAGGGTG	31	63
	19519	Coding	1113	GTGGAACCTGGGCACGGGA	41	64
	19520	Coding	1118	TGAAGGTGGAACCTGGGCA	51	65
	19521	Coding	1127	AGCTGGAGGTGAAGGTGG	59	66
	19522	Coding	1162	CGCAAAGTTGGGACAGTC	80	67
15	19523	Coding	1184	GTGCCACCTCTCTGCGGG	40	68
	19524	Coding	1269	CTGTCTCCCACTTCTGA	67	69
	19525	Coding	1290	AGGCTCTGTGGCTTGTGG	79	70
	19526	Coding	1389	TCGTGGTCGCTCAGCCCT	75	71
	19527	Coding	1465	CCGCCTCCAGGTCGCCAG	57	72
20	19528	Coding	1537	GCAGCCCAGCAGGTCCAT	68	73
	19529	Coding	1545	TCCTCCAGCAGCCACGC	80	74
	19530	Coding	1604	ATCTGAGAAGACTGGGCG	19	75
	19531	Coding	1707	GCTCCTGCTTGCCCTGTC	89	76
	19532	Coding	1732	GTTAGCACCAAGTAGGCG	80	77
25	19533	Coding	1842	CGCAAACCACCACTCAG	79	78
	19534	Coding	1847	ATCCTCGCAAACCACCCA	42	79
	19535	Coding	1859	ATAGCGTCCCTCATCTC	52	80
	19536	Coding	1925	CTCAGGGACGAACCAAGG	92	81
	19537	Coding	1930	AAAGGCTCAGGGACGAAC	41	82
30	19538	Coding	1979	ACAAAACAAAACAAAACA	0	83
	19539	Coding	2016	GCCAAAGTTTCTATTAGTG	87	84
	19540	Coding	2033	GCAGAGGGCACAGGAGTG	59	85
	19541	Coding	2039	GTCCAGGCAGAGGGCACA	72	86
	19542	Coding	2043	GCTTGTCCAGGCAGAGGG	58	87
35	19543	Coding	2071	TGCCTTAGGACAGTTTCTCAG	69	88
	19544	Coding	2085	TCCGTGCTCGCCCTGCC	62	89
	19545	Coding	2089	TTGTTCCGTGCTCGCCCC	57	90
	19546	Coding	2097	AGGCCCCATTGTTCCGTG	79	91

As shown in Table 2, SEQ ID NOs 11, 15, 16, 17, 21, 22,
 23, 28, 29, 30, 31, 33, 35, 36, 37, 38, 39, 41, 43, 46, 47,
 48, 50, 59, 67, 70, 71, 74, 76, 77, 78, 81, 84 and 91

demonstrated at least 75% inhibition of TNFR1 expression in this experiment and are therefore preferred.

Example 18:

Antisense inhibition of TNFR1 expression- phosphorothioate 2'-

5 MOE gapmer oligonucleotides

In accordance with the present invention, a third series of oligonucleotides were designed to target different regions of the human TNFR1, using published sequences (GenBank accession number X55313, incorporated herein as SEQ ID NO: 1, 10 GenBank accession number AA460610, incorporated herein as SEQ ID NO: 92, and GenBank accession number F13533, incorporated herein as SEQ ID NO: 93). The oligonucleotides are shown in Table 3. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which 15 the oligonucleotide binds. All compounds in Table 3 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are 20 composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human TNFR1 mRNA levels by quantitative real-time PCR as 25 described in other examples herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

ORIGINAL TEXT

Table 3

Inhibition of TNFR1 mRNA levels by chimeric
phosphorothioate oligonucleotides having 2'-MOE wings and a
deoxy gap

	ISIS	REGION	TARGET	TARGET	SEQUENCE	%	SEQ
5	26089	5' UTR	1	111	TGAGACTCGGGCATAGAG	39	94
	26091	5' UTR	1	116	AGGGTTGAGACTCGGGCA	43	95
	26092	5' UTR	1	119	TTGAGGGTTGAGACTCGG	59	96
	26093	5' UTR	1	121	AGTTGAGGGTTGAGACTC	11	97
10	26094	5' UTR	1	125	TCACACTTGAGGGTTGAG	42	98
	26096	5' UTR	1	194	AGTGTGGCAGCGCAGTG	35	99
	26099	5' UTR	1	201	TCAGGGCAGTGTGGCAGC	45	100
	26100	5' UTR	1	203	GCTCAGGGCAGTGTGGCA	48	101
	26101	5' UTR	1	205	GGGCTCAGGGCAGTGTGG	39	102
15	26103	5' UTR	1	209	ATTTGGGCTCAGGGCAGT	41	103
	26104	5' UTR	1	211	CCATTITGGGCTCAGGGCA	48	104
	26124	Coding	1	653	TTTCACTCCAATAATGCC	1	105
	26125	Coding	1	655	GTTTTCAC'TCCAATAATG	7	106
	26126	Coding	1	657	AGGTTTTCACTCCAATAA	9	107
20	26127	Coding	1	659	AAAGGTTTTCACTCCAAT	32	108
	26128	Coding	1	671	TGAAGCACTGGAAGAGT	28	109
	26129	Coding	1	673	ATTGAAGCACTGGAAGAG	20	110
	26133	Coding	1	727	GTTCTGTTTCTCCTGGCA	63	111
	26134	Coding	1	729	GTGTTCTGTTTCTCCTGG	52	112
25	26135	Coding	1	731	CGGTGTTCTGTTTCTCCT	70	113
	26136	Coding	1	775	ACACTCGTTTCTCTTAG	20	114
	26137	Coding	1	779	AGACACACTCGTTTCTCT	28	115
	26138	Coding	1	781	GGAGACACACTCGTTTTC	5	116
	26139	Coding	1	803	GGCTTTTCTTACAGTTAC	57	117
30	26140	Coding	1	805	CAGGCTTTTCTTACAGTT	44	118
	26141	Coding	1	846	TTAACATTCTCAATCTGG	11	119
	26142	Coding	1	899	CAAAGAAAATGACCAGGG	0	120
	26143	Coding	1	903	AGACCAAAGAAAATGACC	0	121
	26144	Coding	1	905	AAAGACCAAAGAAAATGA	0	122
35	26145	Coding	1	909	AGGCAAAGACCAAAGAAA	15	123
	26147	Coding	1	915	GATAAAAGGCAAAGACCA	17	124
	26148	Coding	1	917	GGGATAAAAGGCAAAGAC	18	125
	26149	Coding	1	919	GAGGGATAAAAGGCAAAG	11	126
	26150	Coding	1	923	AGAGGAGGGATAAAAGGC	35	127
40	26151	Coding	1	925	GAAGAGGAGGGATAAAAG	0	128
	26152	Coding	1	927	ATGAAGAGGAGGGATAAA	0	129
	26153	Coding	1	931	ACCAATGAAGAGGAGGGA	21	130
	26154	Coding	1	933	AAACCAATGAAGAGGAGG	32	131
	26156	Coding	1	950	GTTGGTAGCGATACATTA	58	132
45	26157	Coding	1	952	CCGTTGGTAGCGATACAT	73	133
	26158	Coding	1	954	CACCGTTGGTAGCGATAC	40	134
	26159	Coding	1	982	ACAAACAATGGAGTAGAG	2	135
	26160	Coding	1	990	GATTTCACCAAAACAATG	34	136
	26161	Coding	1	992	TCGATTTCACCAAAACA	13	137
50	26113	Coding	1	1222	GGCTGTCGCAAGGATGGG	27	138
	26115	Coding	1	1270	GCTGTCTCTCCACTTCTG	19	139

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	26116	Coding	1	1272	GCGCTGTCTCTCCCACTTC	44	140
	26117	Coding	1	1287	CTCTGTGGCTTGTGGGCG	17	141
	26118	Coding	1	1289	GGCTCTGTGGCTTGTGGG	25	142
	26119	Coding	1	1291	TAGGCTCTGTGGCTTGTG	34	143
5	26120	Coding	1	1293	TCTAGGCTCTGTGGCTTG	37	144
	26105	Coding	92	226	TGAAGGACGGTGGAGAGG	2	145
	26106	Coding	92	228	GGTGAAGGACGGTGGAGA	0	146
	26107	Coding	92	230	GAGGTGAAGGACGGTGA	1	147
	26108	Coding	92	231	GGAGGTGAAGGACGGTGG	0	148
10	26109	Coding	92	233	CTGGAGGTGAAGGACGGT	15	149
	26110	Coding	92	235	AGCTGGAGGTGAAGGACG	1	150
	26111	Coding	92	275	GGAGCCGCAAGTTGGTA	11	151
	26112	Coding	92	276	GGGAGCCGCAAGTTGGT	3	152
	26114	Coding	92	332	GAGGCTGTGCGCAAGGATG	14	153
15	26121	Coding	92	495	CTTGGTCGCTCAGCCCTA	26	154
	26122	Coding	92	497	CTCTTGGTCGCTCAGCCC	0	155
	26123	Coding	92	500	GATCTCTTGGTCGCTCAG	13	156
	26130	Coding	93	43	GTCCCATTTGAGCAGAGGC	18	157
	26131	Coding	93	45	CGGTCCCATTTGAGCAGAG	32	158
20	26132	Coding	93	49	TGCACGGTCCCATTGAGC	34	159

As shown in Table 3, SEQ ID NOS 94, 95, 96, 98, 99, 100, 101, 102, 103, 104, 108, 111, 112, 113, 117, 118, 127, 131, 132, 133, 134, 136, 140, 143, 144, 158 and 159 demonstrated at least 30% inhibition of TNFR1 expression in this experiment 25 and are therefore preferred.

Example 19

Real-time Quantitative PCR Analysis of mouse TNFR1 mRNA Levels

Quantitation of mouse TNFR1 mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard 35 PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR 40 primers, and contains two fluorescent dyes. A reporter dye

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(e.g., JOE or FAM, PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, 5 reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of 10 the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is 15 monitored at regular (six-second) intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent 20 inhibition after antisense oligonucleotide treatment of test samples.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25 μ L PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM $MgCl_2$, 300 μ M 25 each of dATP, dCTP and dGTP, 600 μ M of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNase inhibitor, 1.25 Units AMPLITAQ GOLD™, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25 μ L poly(A) mRNA solution. The RT reaction was carried out by 30 incubation for 30 minutes at 48°C. following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD™, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

Probes and primers to mouse TNFR1 were designed to hybridize to a mouse TNFR1 sequence, using published sequence information (GenBank accession number X57796, incorporated herein as SEQ ID NO:160). For mouse TNFR1 the PCR primers

5 were:

forward primer: AAGTATGTCCATTCTAAGAACAATTCCA (SEQ ID NO: 161)
reverse primer: CTCGGACAGTCACTCACCAAGTAG (SEQ ID NO: 162) and
the PCR probe was: FAM- TGCTGCACCAAGTGCCACAAAGGA-TAMRA
(SEQ ID NO: 163) where FAM (PE-Applied Biosystems, Foster
10 City, CA) is the fluorescent reporter dye) and TAMRA (PE-
Applied Biosystems, Foster City, CA) is the quencher dye. For
mouse GAPDH the PCR primers were:

forward primer: GGCAAATTCACGGCAGT (SEQ ID NO: 164)
reverse primer: GGGTCTCGCTCCTGGAAGCT (SEQ ID NO: 165) and the
15 PCR probe was: 5' JOE-AAGGCCGAGAATGGGAAGCTTGTCATC- TAMRA 3'
(SEQ ID NO: 166) where JOE (PE-Applied Biosystems, Foster
City, CA) is the fluorescent reporter dye) and TAMRA (PE-
Applied Biosystems, Foster City, CA) is the quencher dye.

Example 20

20 Northern blot analysis of mouse TNFR1 mRNA levels

Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL RNazol™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA was prepared following manufacturer's recommended protocols. Twenty
25 micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon, OH). RNA was transferred from the gel to HYBOND™-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight
30 capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc, La Jolla, CA).

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Membranes were probed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions with a mouse TNFR1 specific probe prepared by PCR using the forward primer:

5 AAGTATGTCCATTCTAAGAACAATTCCA (SEQ ID NO: 161)

reverse primer: CTCGGACAGTCACTCACCAAGTAG (SEQ ID NO: 162).

To normalize for variations in loading and transfer efficiency membranes were stripped and probed for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) RNA (Clontech, Palo Alto, CA).

10 Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to G3PDH levels in untreated controls.

Example 21

15 **Antisense inhibition of mouse TNFR1 expression-phosphorothioate 2'-MOE gapmer oligonucleotides**

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the mouse TNFR1, using published sequences (GenBank accession
20 number X57796, incorporated herein as SEQ ID NO: 160). The oligonucleotides are shown in Table 4. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 4 are chimeric oligonucleotides
25 ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages
30 are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on mouse TNFR1 mRNA levels by quantitative real-time PCR as described in other examples

herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

Table 4

5 Inhibition of mouse TNFR1 mRNA levels by chimeric
phosphorothioate oligonucleotides having
2'-MOE wings and a deoxy gap

	ISIS	REGION	TARGET	SEQUENCE	%	SEQ
	10840	5' UTR	1	AGAAGGTAGGAGCGGAATTC	9	167
10	10840	5' UTR	40	GTTCCGAAAACCTCGGAGAAA	52	168
	10840	5' UTR	54	GATCATGAGCCAGAGTTCCG	38	169
	10840	5' UTR	62	GTAGGCCCGATCATGAGCCA	57	170
	10840	5' UTR	69	GCACCCAGTAGGCCCGATCA	61	171
	10840	5' UTR	89	GTACAGTCTCCAGGACCTC	27	172
15	10841	5' UTR	110	CAGAGCAGATAGAGATCAG	52	173
	10841	5' UTR	129	AGTTCGAGAAGCTGAAAGTC	51	174
	10841	5' UTR	149	CGATGGCAGCCTGGGCCCTCG	56	175
	10841	5' UTR	169	ATCGGACCCAGGTGGCCCGGG	40	176
	10841	5' UTR	189	CTCGTGAATGAAGTAAAGAT	68	177
20	10841	5' UTR	208	AGGGCAGCAATTGACAACGC	60	178
	10841	5' UTR	258	CCCATGTCGGCCCGGCAGTG	50	179
	10841	Coding	295	CACCAGTGACAGCAGCAGGC	72	180
	10841	Coding	314	CCATCAGCAGAGCCAGGAGC	63	181
	10841	Coding	333	ACCCCTGATGGATGTATCCC	64	182
25	10842	Coding	353	GAGAAGGGACTAGTCCAGTC	46	183
	10842	Coding	373	CCTCTTCTCCCGTCAACAA	74	184
	10842	Coding	410	TAGAAATGGACATACTTTCT	67	185
	10842	Coding	430	GCAGCAGATGGAATTGTCT	79	186
	10842	Coding	458	CCAAGTAGGTTCTCTTGTGG	44	187
30	10842	Coding	487	ATCCCGCCCTGGGCTCGGAC	63	188
	10842	Coding	515	TGCCCCTTTTCACACTCCCTG	86	189
	10842	Coding	543	CTGAGGTAATTTCTGGGAAGC	64	190
	10842	Coding	571	CCGACATGTCTTGCAACTGA	45	191
	10842	Coding	600	GGAGAGATCTCCACCTGGGA	62	192
35	10843	Coding	628	ACACACCGTGTCTTGTCTAG	65	193
	10843	Coding	655	GCGTTGGAAGTGGTTCTCTCT	51	194
	10843	Coding	683	CGCAGTGGAAAGTGTGTCTCA	62	195
	10843	Coding	744	GTGTTCTGAGTCTCTTACAA	74	196
	10843	Coding	772	AAAGAACCCCTGCATGGCAGT	59	197
40	10843	Coding	800	TGCAAGGGACGCACTCACTT	64	198
	10843	Coding	844	AGGTAGGCACAACCTTCATAC	68	199
	10843	Coding	889	CGCAGTACCTGAGTCCTGGG	53	200
	10843	Coding	933	GATAGAAGGCCAAAGACCTAG	59	201
	10843	Coding	960	CGGCACATTAACACTGATGAA	64	202
45	10844	Coding	1005	TCCCTACAAATGATGGAGTA	41	203
	10844	Coding	1032	GCCTTCTCTCTTTTGACAGG	62	204
	10844	Coding	1170	TTACTAGGACCGAAGATGGG	23	205
	10844	Coding	1199	CCTCACTGACAGGTGGCATG	50	206

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of mouse TNFR1 expression in this experiment and are therefore preferred.

Example 22

Effect of TNFR1 Antisense Oligonucleotides in an Endotoxin and 5 D(+)-Galactosamine-induced Murine Model of Fulminant Hepatitis and Liver Injury

The lipopolysaccharide/D-galactosamine or LPS/GalN model is a well known experimental model of toxin-induced hepatitis. Injection of the endotoxin, lipopolysaccharide (LPS), induces
10 septic shock death in the mouse, though with LPS alone, the mouse liver does not sustain major damage. Injection of D-Galactosamine (GalN), while metabolized in liver causing depletion of UTP, is not lethal to mice. It does, however, sensitize animals to TNF- α or LPS-induced endotoxic shock by
15 over 1,000 fold. In the presence of GalN, LPS induces apoptotic cell death in liver, thymus, spleen, lymph nodes and the kidney and results in fulminant death in animals. The liver injury is known to be transferable via the serum, suggesting a mechanism of action under TNF- α control. Further
20 support for this mechanism is provided by the finding that TNFR1 knockout mice are resistant to LPS/GalN-induced liver injury and death.

Eight-week-old female Balb/c mice were used to assess the activity of TNFR1 antisense oligonucleotides in the endotoxin
25 and D(+)-Galactosamine-induced murine model of fulminant hepatitis and liver injury. Mice were intraperitoneally pretreated with 24mg/kg of ISIS 108426 (SEQ ID NO. 189) four times a day for 2 days. Control mice were injected with saline. One day after the last dose of oligonucleotide, mice
30 were injected intraperitoneally with 5ng LPS (DIFCO laboratories) and 20mg D-Galactosamine (Sigma) per animal in saline. At time intervals of 5.5, 7.5, 9.5, 21.5, 30, 45 and

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53 hours after the final dose, animals were monitored for survival rates. Results are shown in Table 5.

TABLE 5

Protective Effects of TNFR1 Antisense Chimeric (deoxy
5 gapped) Phosphorothioate Oligonucleotides in Endotoxin and
D(+)-Galactosamine-induced Murine Model of Hepatitis
and Liver Injury

	Isis #	SEQ	% Survival						
			5.5	7.5	9.5	21.5	30	45	53
	Saline	--	100	100	20	20	10	10	10
10	108426	189	100	100	100	100	100	100	100

The data show that antisense inhibitors of TNFR1 can protect against death in this model of toxin-induced hepatitis. While not wishing to be bound by theory, this is believed to indicate that the biological consequences of TNF 15 activation can be blocked through antisense mediated target reduction of TNFR1 in mouse.

The level of TNFR1 RNA was also measured at intervals of 0, 2, 4, 6, and 9 hours after the last endotoxin challenge. Mice were sacrificed and the livers were removed from the animals and analyzed for TNFR1 mRNA expression. RNA was extracted using the RNEASY™ kit (Qiagen, Santa Clarita, CA) and quantitated by ribonuclease protection assay. Ribonuclease protection experiments were conducted using RIBOQUANT™ kits and the mAPO-2 Custom Probe Set set according to the manufacturer's instructions (Pharmingen, San Diego, CA). mRNA levels were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Target levels of TNFR1 were reduced by 86% in animals treated with ISIS 108426 compared to control saline treated mice.

What is claimed is:

1. An antisense compound 8 to 30 nucleotides in length targeted to a nucleic acid molecule encoding TNFR1, wherein said antisense compound inhibits the expression of TNFR1.

5

2. The antisense compound of claim 1 which is an antisense oligonucleotide.

3. The oligonucleotide of claim 2 comprising at least
10 an 8-nucleobase portion of SEQ ID NO 11, 15, 16, 17, 21, 22,
23, 25, 28, 29, 30, 31, 33, 35, 36, 37, 38, 39, 41, 42, 43, 45,
46, 47, 48, 50, 59, 62, 67, 70, 71, 74, 76, 77, 78, 81, 82, 84,
90, 91, 94, 95, 96, 98, 99, 100, 101, 102, 103, 104, 108, 111,
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191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202,
203, 204, 206, 207, 208, 209, 211, 212, 213, 214, 215, 217,
218, 220, 221, 222, 224, 225, 226, 227, 229, 230, 231, 232,
20 233, 234, 235, 236, 237, 238, 239, 240, 242, 243, 244 or 246.

4. The oligonucleotide of claim 2 comprising SEQ ID NO:
11, 15, 16, 17, 22, 30, 33, 70, 74, 76 and 78.

5. The oligonucleotide of claim 2 which comprises at
least one modified internucleoside linkage.

25

6. The oligonucleotide of claim 5 wherein the modified
internucleoside linkage is a phosphorothioate linkage.

7. The oligonucleotide of claim 2 which comprises at
least one modified sugar moiety.

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8. The oligonucleotide of claim 7 wherein the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.

9. The oligonucleotide of claim 2 which comprises at least one modified nucleobase.

5 10. The oligonucleotide of claim 9 wherein the modified
nucleobase is a 5-methylcytosine.

11. The oligonucleotide of claim 2 which is a chimeric oligonucleotide.

12. A pharmaceutical composition comprising the
10 antisense compound of claim 1 and a pharmaceutically acceptable
carrier or diluent.

13. The pharmaceutical composition of claim 12 comprising a colloidal dispersion system.

14. The pharmaceutical composition of claim 12 wherein
15 the antisense compound is an antisense oligonucleotide.

15. A method of inhibiting the expression of TNFR1 in cells or tissues comprising contacting said cells or tissues with the antisense compound of claim 1 so that expression of TNFR1 is inhibited.

20 16. The method of claim 15 wherein said cells or tissues
are human cells or tissues or murine cells or tissues.

17. A method of treating an animal having a disease or condition associated with TNFR1 comprising administering to said animal a therapeutically or prophylactically effective amount of the antisense compound of claim 1 so that expression of TNFR1 is inhibited.

18. The method of claim 17 wherein said animal is a human.

19. The method of claim 17 wherein the disease or
5 condition is a liver disease.

20. The method of claim 19 wherein the liver disease is hepatitis.

21. The method of claim 17 wherein the disease or
10 condition is liver injury.

22. The method of of claim 17 wherein the disease or condition is a hyperproliferative disorder.

23. The method of claim 22 wherein the hyperproliferative disorder is cancer.

15 24. The method of of claim 23 wherein the cancer is liver cancer.

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ABSTRACT

Antisense compounds, compositions and methods are
5 provided for modulating the expression of TNFR1. The
compositions comprise antisense compounds, particularly
antisense oligonucleotides, targeted to nucleic acids encoding
TNFR1. Methods of using these compounds for modulation of
TNFR1 expression and for treatment of diseases associated with
10 expression of TNFR1 are provided.

2020-11-15-00

SEQUENCE LISTING

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 Ala Ala Leu Pro Pro Ala Pro Ser Leu Leu Arg * 455
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Ser Arg Ser Leu Gly Pro Gly Arg Asp Leu Tyr Ala Arg Val Ser Thr
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Leu Asn Cys His Pro Lys Ala Leu Gly Thr Ser Trp Thr Asp Arg Val
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Pro Gly Ser Pro Ser Thr Ala Ala Ala Thr Leu Pro * Ala Gln Met
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ggg gag tga gag gcc ata gct gtc tgg cat ggg cct ctc cac cgt cct 240
Gly Glu * Glu Ala Ile Ala Val Trp His Gly Pro Leu His Arg Pro
65 70 75
tca cct cca gct cca cct ata ccc cgg gtg act gta cca act ttg cgg 288
Ser Pro Pro Ala Pro Pro Ile Pro Val Thr Val Thr Leu Arg
80 85 90
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Leu Pro Ala Glu Arg Trp His His Pro Ile Arg Gly Leu Thr Pro Ser
95 100 105
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Leu Arg Gln Pro Arg Leu Arg Pro His Pro Gln Pro Pro Ser Glu Val
110 115 120 125
gga gga cag cgc cca caa gcc aca gag cct aga cac tga tga ccc cgc 432
Gly Gly Gln Arg Pro Gln Ala Thr Glu Pro Arg His * * Pro Arg
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140 145 150 155

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 Ala Gln Trp Asp Arg Ala Pro Leu Leu Pro Gly Glu Thr Glu His Arg
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 Val His Leu Pro Cys Ser Xaa Leu Ser Lys Arg Lys Arg Val Cys Leu
 35 40 45

ctg tng taa ctg taa gaa aag cct ggn gtg cac gaa gtt gtg cct acc 192
 Leu Xaa * Leu * Glu Lys Pro Gly Val His Glu Val Val Pro Thr
 50 55 60

cca gat tga gaa tgt taa ggg cac tga ggn ctc agg cac cac agt gct 240
 Pro Asp * Glu Cys * Gly His * Gly Leu Arg His His Ser Ala
 65 70 75

gtt gcc cct ggt cat ttt ctt tgg tct ttg cct ttt atc cct cct ctt 288
 Val Ala Pro Gly His Phe Leu Trp Ser Leu Pro Phe ile Pro Pro Leu
 80 85 90

cat tgg ttt aat gta tcg cta cca acg gtg gaa gtc caa gct cta ctc 336
 His Trp Phe Asn Val Ser Leu Pro Thr Val Glu Val Gln Ala Leu Leu
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Figure 6

[illegible]

Figure 6

Figure 6

Docket No.
ISPH-0518

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

ANTISENSE MODULATION OF TNFR1 EXPRESSION

the specification of which

(check one)

☒ is attached hereto.

☐ was filed on _____ as United States Application No. or PCT International Application Number _____ and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)

(Filing Date)

(Application Serial No.)

(Filing Date)

(Application Serial No.)

(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

PCT/US99/13763

June 17, 1999

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

09/106,038

June 26, 1998

patented

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)
(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

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